

FORM PTO-1390 (Modified)  
(REV 11-2000)

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

101137-31

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

09/980646

INTERNATIONAL APPLICATION NO.  
PCT/NL00/00268INTERNATIONAL FILING DATE  
26 April 2000 (26.04.00)PRIORITY DATE CLAIMED  
26 April 1999 (26.04.99)

## TITLE OF INVENTION

Identification of Ligands for Orphan Receptors Using Mass Spectrometry

## APPLICANT(S) FOR DO/EO/US

Jan van der Greef; Hubertus Irth

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau)
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
  - a. ☐ is attached hereto.
  - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A copy of the International Search Report (PCT/ISA/210).

## Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☐ Other items or information:

101137-31

CALCULATIONS PTO USE ONLY

<input type="checkbox"/>	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . .	<b>\$1040.00</b>
<input checked="" type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . . .	<b>\$890.00</b>
<input type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO . . . . .	<b>\$740.00</b>
<input type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . .	<b>\$710.00</b>
<input type="checkbox"/>	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . .	<b>\$100.00</b>

**\$890.00**

**\$130.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	11 - 20 =	0	x \$18.00
Independent claims	2 - 3 =	0	x \$84.00
Multiple Dependent Claims <b>(check if applicable)</b> .			<input type="checkbox"/>

<b>TOTAL OF ABOVE CALCULATIONS</b>	<b>=</b>	<b>\$1,020.00</b>
------------------------------------	----------	-------------------

☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

**\$0.00**

<b>SUBTOTAL</b>	<b>=</b>	<b>\$1,020.00</b>
-----------------	----------	-------------------

Processing fee of **\$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

**\$0.00**

<b>TOTAL NATIONAL FEE</b>	<b>=</b>	<b>\$1,020.00</b>
---------------------------	----------	-------------------

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable)**.

**\$0.00**

<b>TOTAL FEES ENCLOSED</b>	<b>=</b>	<b>\$1,020.00</b>
----------------------------	----------	-------------------

Amount to be: refunded	\$
charged	\$

- a. ☐ A check in the amount of \_\_\_\_\_ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 14-1263 in the amount of \$1,020.00 to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment  
to Deposit Account No. \_\_\_\_\_ A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card  
information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

correspondence address associated with Customer No.27387

SIGNATURE \_\_\_\_\_

**Bruce S. Londa**

NAME \_\_\_\_\_

33.531

REGISTRATION NUMBER

**October 26, 2001**

DATE \_\_\_\_\_

09/980646

JC10 Rec'd PCT/PTO 26 OCT 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty's Docket No. 101137-31

APPLICANT : Jan van der Greef et al.  
FILED : Concurrently Herewith  
FOR : Identification of Ligands for Orphan Receptors  
Using Mass Spectrometry

PRELIMINARY AMENDMENT

Hon. Assistant Commissioner of Patents  
Washington, D.C. 20231

Sir:

Prior to examination, please amend the application as  
follows:

**IN THE SPECIFICATION**

Page 1, after line 2, please insert --Background of the  
Invention--;

Page 4, after line 25, please insert --Summary of the  
Invention--;

Page 5, after line 13, please insert  
--Brief Description of the Drawings  
Fig. 1 is a graft representing a typical MS spectrum of digoxin;

Fig. 2 is a graft representing the base peak in MS/MS spectra with wideband activation of the protonated molecule (780.8 +/- 18) at 45% collision energy which fragmentates both the parent mass as well as the ammonium complex 60 651.1.

Detailed Description of the Preferred Embodiments--.

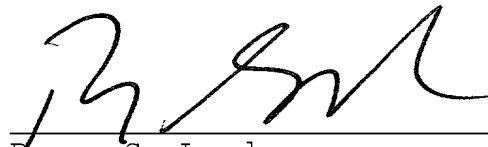
#### IN THE CLAIMS

Please amend the claims in accordance with the attached marked-up pages. A clean copy of the amended claims is also enclosed.

#### REMARKS

The above amendments were made to place the application into proper United States Patent Format.

Respectfully Submitted,



Bruce S. Londa  
Attorney for Applicant  
Norris, McLaughlin & Marcus P.A.  
220 East 42<sup>nd</sup> Street, 30<sup>th</sup> Floor  
New York, N.Y. 10017  
Telephone: (212) 808-0700  
Telecopier: (212) 808-0844

## Claims - Marked-up Copy

1. On-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule to said effluent, whereby the affinity molecules bind analytes in the effluent, followed by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.

2. On-line detection method according to claim 1, in which the second separation step is carried out using a restricted-access support, in which the affinity molecule is retained, followed by elution of the analyte from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer.

3. On-line detection method according to claim 1, in which the second separation step is carried out using a hollow fiber

Claims - Marked-up Copy

support, whereby the analyte is permeated and the permeate is directed to the mass spectrometer.

4. (amended) ~~On-line detection method according to any of the preceding claims claim 1,~~ in which the dissociation step is a low pH shock, contacting with a high ionic strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.

5. (amended) ~~Method The method according to any of the preceding claims claim 1,~~ in which the fractionation step is a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system, which is optionally followed by a separation step which removes the high molecular weight background.

6. (amended) ~~Method The method according to claim 5,~~ in which the liquid chromatography separation step is a HPLC, a reversed phase HPLC, a CE, a CEC, a IEF or a MEKC step.

7. (amended) ~~Method The method according to any one of the preceding claims claim 1,~~ in which the mass spectrometer is of the type chosen from the group consisting of electrospray

Claims - Marked-up Copy

ionization type, atmospheric pressure ionization type, quadrupole type, magnetic sector type, time-off flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combination thereof.

8. (amended) ~~Method~~ The method according to any one of the preceding claims claim 1, in which the mass spectrometer is set to detect ions of a selected single m/z trace, selected multiple m/z traces, in scanning mode or any sequential mode.

9. (amended) ~~The method of any of the preceding claims~~ according to claim 1, wherein the affinity molecule is an affinity protein.

10. (amended) ~~The method of any of the preceding claims~~ according to claim 1, wherein the affinity molecule is an orphan receptor.

11. (amended) ~~Compound detected by the method of any one of the preceding claims~~ claim 1.

12. ~~The use of a compound of the preceding claims as a ligand for affinity molecules.~~

## Claims - Clean Copy

1. On-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule to said effluent, whereby the affinity molecules bind analytes in the effluent, followed by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.

2. On-line detection method according to claim 1, in which the second separation step is carried out using a restricted-access support, in which the affinity molecule is retained, followed by elution of the analyte from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer.

3. On-line detection method according to claim 1, in which the second separation step is carried out using a hollow fiber



Claims - Clean Copy

support, whereby the analyte is permeated and the permeate is directed to the mass spectrometer.

4. (amended) On-line detection method according to claim 1, in which the dissociation step is a low pH shock, contacting with a high ionic strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.

5. (amended) The method according to claim 1, in which the fractionation step is a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system, which is optionally followed by a separation step which removes the high molecular weight background.

6. (amended) The method according to claim 5, in which the liquid chromatography separation step is a HPLC, a reversed phase HPLC, a CE, a CEC, a IEF or a MEKC step.

7. (amended) The method according to claim 1, in which the mass spectrometer is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, magnetic sector type, time-off

Claims - Clean Copy

flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combination thereof.

8. (amended) The method according to claim 1, in which the mass spectrometer is set to detect ions of a selected single m/z trace, selected multiple m/z traces, in scanning mode or any sequential mode.

9. (amended) The method according to claim 1, wherein the affinity molecule is an affinity protein.

10. (amended) The method according to claim 1, wherein the affinity molecule is an orphan receptor.

11. (amended) Compound detected by the method of claim 1.

09-980,646  
PTO/PCT Rec'd 26 OCT 2001

Title:

IDENTIFICATION OF LIGANDS FOR ORPHAN RECEPTORS USING MASS SPECTROMETRY

The present invention relates to the on-line coupling of mass spectrometry (MS) to continuous-flow separation techniques for detecting 'orphan' analytes, *viz.* affinity molecules which do not have known ligands, such as orphan affinity proteins, *e.g.* orphan receptors. In a further  
5 embodiment, this on-line detection method can be used as a screening method for assays with an unknown ligand. Furthermore, this invention relates to compounds detected by this method and the use of these compounds as a ligand for affinity molecules.

A range of biochemical assays based on continuous flow biochemical  
10 detection has been developed during the recent years. Typically, the affinity interaction between ligand and affinity protein has been monitored by measuring the concentration of highly sensitive, usually fluorescence labeled reporter molecules using either heterogeneous or homogeneous biochemical detection strategies. When dealing with 'orphan' receptors however, *i.e.*  
15 receptors for which 'affinity' ligands have not been found so far, new strategies have to be developed, which enable so-called 'label-free detection' of affinity ligands. In contrast to the label-based assays, which usually allow the non-binding analytes from the sample to co-elute with the reporter molecules, label-free assays for orphan receptors demand strict separation of both binding  
20 and non-binding ligands.

By configuring restricted access columns and hollow fiber modules in the biochemical part of the system, affinity ligands can be isolated from complex biological matrices. Subsequently, coupling the biochemical setup to mass spectrometry allows the on-line detection of ligands exhibiting affinity  
25 for the orphan affinity molecule.

It is known from the prior art that biochemical assays are highly sensitive detection techniques, which combine the selectivity of biospecific interactions with the sensitive detection of labels used as reporter molecules.

An example of such a technique is described in WO 91/13354. This  
30 document describes a flow immunosensor wherein an antibody specific to an affinity molecule is immobilized on a support. The antibody's binding sites are saturated with a labeled form of a known ligand. A liquid to be analyzed is contacted with the antibody to allow any affinity molecule present in the liquid

to displace the labeled antigen. Finally, the displaced labeled antigen is detected.

However, it is known as well that biochemical assays suffer from the problem of cross-reactivity, which means that antibodies react with more than one analyte. This leads to erroneous results. For this reason, biochemical assays are frequently combined with a fractionation step, *e.g.*, a separation step using HPLC or another type of liquid chromatography.

In the field to which the present invention relates, there is a demand for on-line coupling of a fractionation step and an biochemical assay detection system. Several approaches have been proposed and described to perform continuous-flow biochemical assays. Most of these biochemical assays are in the form of a postcolumn reaction detection system based on a sequential addition type biochemical assay.

Cassidy *et al.*, *Anal. Chem.* 64 (1992), 1973-1977 disclose a kinetically controlled immunoassay based on the sequential addition of antibody, sample and label on a protein A column, performing immunoassays for albumin and transferrin in less than 1 minute.

Nilson *et al.*, *J. Chromatography*, 597 (1992), 383-389 describe a continuous-flow competitive assay involving enzyme-labeled antibodies. The system described was coupled to a size-exclusion column to allow the monitoring of activity.

These prior art techniques which are of a sequential nature are not suitable for on-line coupling to liquid chromatographic or other fractionation systems, since these techniques do not allow the continuous monitoring of the fractionation effluent.

In an article in *Analytical Chemistry* 68 (1996), 4226-4236, Nedved *et al.* describe a method to characterize benzodiazepines. The method encompasses the binding of benzodiazepines to antibodies on a protein-6-column. The complexes formed are eluted using a pH-step. The benzodiazepines are separated from the antibodies by using a restricted access (RA) column, leaving the benzodiazepines in the column material. After this three step method that cannot be used in a continuous process, the benzodiazepines are eluted from the RA column and transported to a mass spectrometer for detection.

Siegel *et al.* Describe in *J. Mass Spectr.* 33 (1998), 264-273 a method wherein a sample containing protease and inhibitors is incubated for 1 hour

and subsequently the sample is subjected to a separation on molecular weight. This separation step can be carried out using a gel-permeation chromatography technique or a microconcentration technique. The method is not suitable for a continuous detection method.

5 Neither suitable for an continuous on-line detection technique is the method described by Dunayevskiy *et al.* in Rapid Communications in Mass Spectrometry 11 (1997), 1178-1184. The method requires a gel-filtration column to encapture unbound components and to pass proteinaceous material.

10 Blom *et al.* described in J. Combinatorial Chem. 1 (1999), 82-90 a method wherein a mixture of protein/ligand complexes and unbound ligand are separated on a size exclusion column, wherein the complexes are caught in a protein trap. From this trap the complexes are eluted by means of a gradient LC pump. The effluent is led to a MS.

15 Neither a suitable continuous on-line method is described by Huyer *et al.* in Analytical Biochemistry 258 (1998), 19-30 involving separation of peptide-enzyme complexes from unbound enzymes using gel-filtration spin columns, followed by on-line concentration using a C<sub>18</sub>-cartridge and injection in a MS.

20 In the article of Irth *et al.* in the Journal of Chromatography 633 (1993), 65-72 and in the articles of Oosterkamp *et al.* in Anal. Chem. 66 (1994), 4295-4301, and in the Journal of Chromatography 653 (1994), 55-61, a method for the on-line detection of digoxigenin and its metabolites is described. The on-line detection process comprises the direct injection of a sample containing digoxigenin and its metabolites, a liquid chromatographic (LC) fractional  
25 separation step, the mixing of the effluent of the LC column with fluorescein-labeled antibodies against digoxigenin, the removal of free labeled antibodies from the mixture via passage through a small column packed with an antigen-bound support, and detection of the strongly fluorescent biochemical complexes.

30 The digoxigenin system described is based on association reactions of antibodies and antigens eluting from the analytical column. By the use of fluorescein-labeled antibodies detection limits in the nanomolar range are obtained.

35 This prior art assay is a heterogeneous detection system. It requires a separation step between free and bound label. Examples for free/bound label separation techniques are restricted-access phases and hollow-fibre modules.

The biochemical reagent in the previously described prior art assay consists of fluorescein-labeled fragments of anti-digoxigenin antibodies which were immunopurified and are commercially available. The commercial availability of purified, labeled antibodies is however exceptional. In almost all cases, antibodies are only available in unlabelled state in crudely purified antiserum. Although labeling and purification schemes for antibodies (or other affinity proteins) are known to the person skilled in the art, it will be preferred to use antisera, which may be commercially available, without any pretreatment.

Hsieh *et al.* (Molecular Diversity, 2 (1996), 189-196), describe a screening method using chromatography coupled with mass spectrometry. Their method requires a dual run, first in the absence of a library of protein targets and then with the library. This means that background compounds, especially the affinity proteins causing severe background signals over a broad range in the mass spectrum, are always present in this assay type, which limits the efficiency, selectivity and flexibility of the method considerably.

Kaur *et al.* (Journal of Protein Chemistry 16, No 5 (1997)), describe a method in which active components in a combinatorial library are bound to a receptor, isolated by size exclusion chromatography and trapped on a column after which the desorption solvent is used to dissociate the affinity protein/ligand complex. Subsequent identification is done by mass spectrometry, but with a background of the affinity protein.

The methodology described by Hsieh and Kaur is thus hampered by severe background signals in the mass spectrometer of the present affinity protein.

One of the advantages of the present invention is that it provides the possibility to detect the unknown ligand in the absence of a protein background in the MS. Especially for low concentration this is a requirement for successful assays.

One aim of the invention is to provide an improvement on the known assays by providing a technique, which does not require the use of known ligands.

The above mentioned problems are overcome by the provision of an on-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule, such as an affinity

protein, to said effluent, whereby the affinity molecules bind analytes in the effluent, followed by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.

The use of the first restricted access column provides the permeation of the analyte-affinity molecule complex while the smaller molecules are retained.

The dissociation step results in the dissociation of the complex. By subsequently separating the affinity molecules from the effluent the analyte can be directed to the MS.

In particular, a suitable affinity molecule for analytes to be detected - which affinity molecule may e.g. be an affinity protein - is added to the effluent of a liquid chromatography or a capillary electrophoresis column to react with the analytes eluting from the column.

The second separation step is carried out either by using a restricted-access support or by using a hollow fiber support.

When using a restricted-access support in the second separation step the affinity molecule is retained, followed by elution of the affinity molecule from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer. Prior to elution of the affinity molecule, a washing step can be carried out to remove the dissociation solvent and the receptor proteins from the restricted-access column. In order to prevent false positives, it is important that the conditions, with respect to concentrations of compounds such as solvents, of the second restricted-access column are substantially constant and substantially the same as in the first restricted-access column.

When using a hollow fiber support in the second separation step the ligand is permeated and the permeate is directed to the mass spectrometer.

Preferably the dissociation step is a low pH shock, such as injection of a hydrochloric acid solution with a pH below 2.5, contacting with a high ionic strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.

The fractionation step according to the present invention can be a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system, which is optionally followed by a separation step which removes the high molecular weight background.

5 Preferred liquid chromatography fractionation steps comprise HPLC, reversed phase HPLC, capillary electrophoresis (CE), capillary electrochromatography (CEC), isoelectric focusing (IEF) or micellar electrokinetic chromatography (MEKC), all of which techniques are known to the person skilled in the art.

10 All known MS options and techniques can be used. Preferably the mass spectrometer is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, magnetic sector type, time-off-flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combinations thereof.

15 The present invention enables the detection of ligands for orphan receptors. Therefore the present invention is also directed to any such new ligand, found with the method set out in the above, as well as the use of these ligands as a ligand for orphan receptors.

20 The combination of the fractionation step with the MS detection step in accordance with the present invention greatly enhances the performance of both techniques. The combined techniques provide an analytical method, which is characterized by a high selectivity and a high sensitivity. Further, the problem associated with cross-reactivity does not occur when using the method of the present invention.

25 The method of the present invention uses bioaffinity molecules such as receptors, to detect any compounds showing high affinity for the ligand binding site of said affinity molecule. The compounds to be detected may be biochemical compounds but are not in any way restricted thereto.

30 A system of high-throughput screening to be used in drug discovery may, for instance, comprise the following steps. Complex samples generated for instance by an upstream combinatorial chemistry system are prefractionated in fractions containing compounds of similar polarity using, e.g., a solid-phase extraction technique or electrophoretic sample handling principles. Each fraction may additionally be fractionated using, e.g., either  
35 analytical or preparative-scale liquid chromatographic fractionating columns. The compounds eluting from said LC column are on-line detected using a



suitable affinity molecule detection technique. Where preparative-scale fractionating columns are applied, a post-column flow-split will be made. One of the two flow streams is subjected to detection using the affinity molecule detection technique; the other stream is directed to a fraction collector.

- 5 Dependent on the signal obtained from the affinity molecule detector, fractions containing compounds causing a positive response will be collected while fractions causing a negative response will be discarded. This complete screening method can be automated using known valve-switching processes.

- 10 A suitable fractionation method to be used in the methods of the present invention comprises a liquid chromatography fractionation or a capillary electrophoresis step. Other fractionation techniques which are known to the person skilled in the art and which allow a relatively continuous output stream can, however, be used as well.

- 15 In a preferred embodiment, the liquid chromatography separation step is a reversed phase HPLC step.

- 20 It will be understood that according to the present invention as the effluent of a fractionation step is also to be understood the effluent of a flow injection, in which a mixture of different compounds is injected, optionally followed by a trapping step, for example using a restricted-access column. The separation step of the method according to the present invention then fulfills the fractionation, since the compounds of interest are fractionated by the separation step. The required selectivity can be obtained by operating the MS selectively tracing of the MS.

- 25 Since the flow injection technique provides a flexible and fast screening method, it is a preferred embodiment of the present invention.

All modes of MS-operation are possible in Flow Injection mode, however, due to the higher background, especially the very selective modes which comprise detecting ions of a selected single  $m/z$  trace or of selected multiple  $m/z$  traces are suitable.

- 30 Restricted-access supports are well-known to the person skilled in the art. In general, restricted-access supports, such as C<sub>18</sub>-silica (for example Lichrospher™ C18 ADS from Merck) packed columns, are capable of retaining smaller sized molecules while permeating the larger molecules. The type of restricted-access supports and the conditions under which they are operated, will depend on the type of assay. A person skilled in the art will be able to
- 35

perform routine experiments to establish the optimal conditions and materials for each case.

Hollow fiber materials, for example made of polysulphon (obtainable from Amicon, Ireland) are well-known to the person skilled in the art. In general, hollow fiber modules are capable of permeating smaller sized molecules while retaining the larger molecules. Also the type of hollow fiber supports and the conditions under which they are operated, will depend on the type of assay. Again, the person skilled in the art will be able to perform routine experiments to establish the optimal conditions and materials for each case.

As already mentioned, the fractionation step can be a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system. It has been found that it is very advantageous when this fractionation step is followed by a separation step, for example by using a hollow-fiber module, since this removes the high molecular weight background. Preferred liquid chromatography fractionation steps comprise HPLC, reversed phase HPLC, CE, CEC, IEF or MEKC, all of which techniques are known to the person skilled in the art.

All possible variations in MS techniques known in the art can be profited from according to the present invention. preferably, the MS is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, triple quadrupoles (QQQ) type, magnetic sector type, time-off-flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combinations thereof.

For example, in scanning mode to trace compounds, low resolution MS with all possible instrumental designs of MS can be used, in particular quadrupole, magnetic sector, time-off-flight, FTMS and ion-trap. This generates typically molecular weight data with nominal mass accuracy.

When high resolution MS is applied, using all possible high resolution instrumental designs, in particular magnetic sector, time-off-flight, FTMS and ion trap, molecular weight data with high mass accuracy combined with the elemental composition of the compound can be obtained.

Other known MS techniques comprise tandem MS, such as MS/MS or MS<sup>n</sup> (for example MS<sup>3</sup>). Application of these techniques enables the collection of structural information of the ligands, which is a preferred embodiment of the present invention. The data in scanning mode can be

acquired in data-dependent mode which means that for each peak observed automatically the tandem MS measurement is performed. In addition to this the fragmentation induced in the tandem MS measurement can be coupled by more sophisticated procedures, for example a broad band excitation, which also  
5 fragments expected fragments of less importance, such as the [protonated molecule-H<sub>2</sub>O]<sup>+</sup> peak in natural product screening.

The MS can also be operated in single/multiple ion monitoring mode, which can be used for highly selective detection.

Single/multiple ion monitoring mode can be used for highly selective  
10 detection. Using low resolution MS in single/multiple ion monitoring mode with all possible low resolution instrumental designs, in particular quadrupole, magnetic sector, time-off-flight, FTMS and ion trap, selective detection based on monitoring of previously determined m/z trace can be obtained. When high resolution MS is applied all possible instrumental  
15 designs of MS can be used, especially quadrupole, magnetic sensor, time-off-flight, FTMS and ion-trap. This enables typically very selective detection based on monitoring in high resolution previously determined m/z trace. When tandem MS is applied, all possible instrumental designs, in particular ion trap, quadrupole-time-off-flight (Q-TOF), FTMS and combinations of sector  
20 instruments with quadrupoles and ion traps, can be used. This enables the very selective detection based on monitoring a resulting peak or peaks in MS/MS and/or MS<sup>n</sup> measurements.

The assays according to the present invention can be based on continuous monitoring of the HPLC effluent after a separation step, for  
25 instance with a hollow fiber step, to remove the high molecular weight background. The MS is typically set to operate in one of single/multiple ions mode options.

With the assays according to the present invention the tracing of (bio-)active compounds in solutions of complex nature, for instance biological  
30 fluids or extracts, natural product extracts, solutions or extracts from biotechnological processes, resulting from chemical experiments, such as combinatorial technologies and processes and the like can be performed with a higher efficiency, selectivity and flexibility. Moreover, the present invention provides the possibility to limit the disturbance of background compounds.

The present invention further enables the identification of compounds based on conventional mass spectra, high resolution data or MS<sup>n</sup> based spectra.

5 With the method of the present invention it is also possible to perform library searching, based on a variety of mass spectrometric experiments enabling the screening of large series of samples and classifying these in classes based on similar active compounds, without the need for full identification of the compounds.

10 The assay method of the present invention can be applied in miniaturized formats of assay-based systems, for instance with chip technology based screening systems.

In the methods of the present invention all molecules capable of interaction with or binding to other molecules can be used as affinity molecule.

15 On-line coupling as used in the methods of the present invention requires fast reaction times in order to minimize extra-column band broadening. This means that affinity molecule-ligand interactions having reaction times in the order of minutes rather than hours should be considered. The suitable binding conditions under which the affinity molecules bind to the analyte comprise a contact time, which is in the same order of magnitude.  
20 Suitable binding conditions are conditions that provide optimal binding between the affinity molecules and the analyte. It will be understood that the precise conditions, such as temperature, residence time, chemical composition, will depend strongly on the type of assay and the proteins used therein.

25 The present invention will be described in further detail while referring to the following examples.

### Example 1 Restricted-access separation

30 Phosphate buffered saline (PBS) carrier phase solution (PBS), affinity protein solution, dissociation solution and desorption solution were pumped by Knauer ministar pumps. The flushing solution was added using a Minipuls 3 peristaltic pump. Two Rheodyne sixport valves were used to enable flushing and column switching. A third rheodyne injection valve was used to perform the loop injections (5 µl injection volume). The carrier phase consisted  
35 of PBS buffer (pH 7.0) which was pumped at a flow rate of 40 µl/min. A solution of anti digoxigenin Fab fragments (5 µM) in PBS was added to carrier

phase at 80  $\mu$ l/min. During control experiments the anti digoxin Fab fragments were switched with either PBS or PBS solutions containing 2  $\mu$ M of goat antibiotin. The dissociation solution consisted of 1 M hydrochloric acid which was added at 260  $\mu$ l/min. The desorption buffer consisted of 80% MeOH and 2 mM ammonium acetate. The rinsing solution was composed of 5% isopropanol/2 mM ammonium acetate and was used to flush the second RA-column for one minute after trapping at 300  $\mu$ l/min. The dimensions of the first and second restricted access columns equaled 4 x 15mm and 2 x 10 mm respectively. The RA-material used was Lichrospher C18 ADS (Merck).

Reactions were carried out in knitted open tubular Teflon reaction coils (0.5 mm ID), using reaction times of 30 s and 30 s for the first and second biochemical reaction respectively. Detection was carried out by a Finnigan LCQ mass spectrometer.

After injection in the carrier phase, digoxin was allowed to react for 30 s with anti digoxigenin Fab fragments. After reaction Fab bound digoxin was able to pass the first restricted access column, whereas excess unbound molecules were trapped onto the first RA-column. After passing the first RA-column the affinity complex was dissociated by a pH shock using a solution of hydrochloric acid. Again the mixture was allowed to react for 30 s after which the ligand could be trapped onto the second RA-column. Three minutes after injection, the second RA-column was flushed for one minute, after which the second RA-column was switched into the desorption solution. Digoxin was desorbed and subsequently detected by the MS.

Figure 1 presents a typical MS spectrum of digoxin. Apart from the protonated molecule (780.8), an ammonium cluster can be observed at 797.9, whereas the m/z ratios of 762.8 and 651.1 represent the loss of respectively water and a sugar group. Wideband activation of the protonated molecule (780.8 +/- 18) at 45% collision energy fragmentates both the parent mass as well as the ammonium complex to 651.1, which can be observed as the base peak in MS/MS spectra under the conditions used (figure 2). During assay operation digoxin was detected using both MS and MS/MS (m/z 797.9 and 651.1 respectively).

Injecting 5, 50 and 500 ng digoxin in the assay system with and without anti digoxigenin Fab fragments showed that digoxin was completely trapped when the affinity protein was absent. However addition of anti digoxigenin Fab fragments showed increasing response of digoxin (measured

at  $m/z$  651.1), demonstrating the ability of the system to detect digoxin using the MS-based orphan assay.

In order to check the robustness of the ms-bcd system, the anti-digoxigenin Fab fragment solution was replaced by goat antibiotin. Injection of 0, 5, 50 and 500 ng digoxin showed that the signal obtained for 500 ng is still comparable to the blank, meaning that the excess of digoxin is adequately trapped on the first restricted access column.

### Example 2 Hollow fiber separation

10

The set-up consisted of two Pharmacia (Uppsala, Sweden) P3500 pumps which delivered the mobile phase and affinity protein solution, one Gilson 306 LC-pump which delivered an acidic solution and two Gilson Minipuls 3 peristaltic pumps which respectively created backpressure at the retentate outlet and added phosphate buffer to the permeate phase. Mobile phase, affinity protein solution and acidic solution were pumped at a flow-rate of 0.2 ml/min. Acidic conditions in the permeate phase were neutralized by the addition of a phosphate buffer (100  $\mu$ l/min). Injections were performed by an Aspec autosampler equipped with two Rheodyne six port valves (injection volume 20  $\mu$ l). A Finnigan LCQ mass spectrometer was used for detection.

20

0.15 ml/min PBS carrier (136.9 mmol/l NaCl, 2.7 mmol/l KCl, 9.2 mmol/l  $\text{Na}_2\text{HPO}_4$  and 1.8 mmol/l  $\text{K}_2\text{PO}_4$ ) comprising eighty randomly chosen drugs, each in the 1  $\mu$ M range, was mixed with 10 nM goat antibiotin (0.15 ml/min, obtained from Pierce, Rockford, Illinois, USA) by means of an inverted Y-piece. The mixture is allowed to react for 48 seconds in a reaction coil prior to the separation of unbound ligands and affinity complexes by a restricted-access column (C18 alkyl diol, Merck Lichrospher<sup>TM</sup> ADS, 15 x 4 mm). Subsequently after passing the restricted access column the pH of the mobile phase is lowered to 2 by addition of hydrochloric acid (0.3 ml/min), causing the affinity complex to dissociate in a reaction coil within 10 s reaction time. On-line filtration by means of a hollow fiber module (polysulphon, obtained from Amicon, 0.5 mm I.D., 30 kDa cut-off, Module I.D. was 0.85 mm) then separates affinity molecules from ligands. The ligands could all subsequently be detected with the MS.

30

### Example 3 Detection of digoxin and related compounds in natural extracts

5 This Example demonstrates the applicability of the MS based orphan bioassay method to rapidly determine ligands for orphan receptors, using a large set of natural extracts spiked with digoxin and structurally related molecules as model compounds. Unbiased determination of bioactive compounds using several software supported background subtraction options  
10 is shown.

**Bioassay description** Affinity protein was added to a stream comprising the analytes. This mixture was then introduced in an open tubular knitted reaction coil, which allowed the affinity protein and sample compounds to interact for 30 s. After the affinity interaction, the mobile phase was led  
15 over a restricted access column in order to separate affinity complexes and non-bioactive compounds. The nature of the restricted access material enables the 'bulky' affinity complexes to pass the column unretained, whereas smaller unbound compounds are trapped inside the hydrophobic pores of the material.

After passing the first restricted access column the affinity  
20 complexes are dissociated by introducing a pH shock, i.e. adding a fairly acidic solution, which lowers the pH to 2. This dissociation reaction proceeds in only 10 s. Subsequently the mobile phase is led over a second restricted access column, which traps the bioactive compounds, whereas the affinity proteins are efficiently removed. Next the restricted access column is flushed with  
25 MilliQ water in order to remove excess of background ions and interfering compounds, after which the RA-column is switched into the desorption solution. Compounds trapped onto the RA-column elute and are introduced into the DECA mass spectrometer, which detects and characterizes the presence of bioactive compounds.

**Biochemical assay configuration** The biochemical assay part of the system consisted of five Knauer ministar LC-pumps. One LC-pump delivered the carrier phase, 10 mM TRIS and 10% DMSO pH 7.5, at a flow rate of 200 µl/min. A second LC-pump delivered 'plugs' of affinity protein, solved in carrier solution, at a maximum flow rate of 100 µl/min. The  
30 dissociation solution, consisting of 10% acetonitrile and 0.25%TFA, was pumped by a third LC-pump at a flow rate of 200 µl/min. In addition the

second restricted access column, was flushed after each injection with MilliQ water for one minute at a flow rate of 1 ml/min. The fifth LC-pump delivered the desorption phase, 75% methanol and 2 mM ammonium acetate, and was operated at a flow rate of 100 µl/min which was directly introduced into the DECA mass spectrometer.

In order to separate affinity complexes from non-bioactive compounds present in the sample a 2\*15 mm C18 ADS restricted access column was used. The second restricted access column, used to separate affinity protein from active compounds, consisted of similar material however differed in dimensions (2\*5 mm).

The reaction coils used were knitted, open tubular, Teflon coils, with an internal diameter of 0.5 mm. The lengths of the reaction coils were chosen in such a way as to allow a 30 and 10 s reaction time for respectively the affinity interaction and the dissociation reaction.

**Specifications mass spectrometer** All measurements were performed on a Finnigan DECA ion trap mass spectrometer, which was equipped with an ESI probe. As the amount of information regarding potential interesting compounds is usually limited, the apparatus was tuned in such a way as to obtain acceptable sensitivity over a broad m/z range. Experiments were run using positive electrospray ionisation and a capillary temperature of 170 °C. The mobile phase, which originates from the biochemical assay part of the system, consisted of 75% of methanol and 2 mM ammonium acetate and was introduced at a flow rate of 100 µl/min.

**Data evaluation** Using Xcalibur 1.1, the operating software of the DECA mass spectrometer, the data was evaluated by performing several software operations on the collected dataset. First the raw datafile was corrected for the background by performing a simple background subtraction routine on the samples. For this purpose several 'blank' samples were included when measuring a series of natural extracts.

After removing the background the corrected MS-spectrum was analysed for active compounds by generating reconstructed ion current spectra (RIC) for those m/z values, which still exhibited a relatively high intensity, meaning an intensity higher than the apparent noise level. By determining the intensity ratio of the m/z value between that observed in the blank sample and that measured in the natural extract, active compounds could be traced easily.



**Natural extract screening** Several natural extracts ranging from fungal to higher plant extracts were spiked with digoxin and cross-reactive compounds, *i.e.* gitoxin, 3, 12-diacetate digoxigenin, digoxigenin and gitoxigenin, in order to demonstrate the applicability of the method in  
5 discovering ligands for orphan receptors in complex extracts. The extracts were spiked with several concentrations of the bioactive compounds. The affinity protein used to simulate the interaction between orphan receptor and ligand was anti digoxigenin Fab, which exhibits reactivity for a range of digoxin related molecules.

10

## Results

**Natural extract screening** Natural extracts, dissolved in 100% DMSO, were tenfold diluted with carrier solution in order to reduce the DMSO  
15 concentration to an acceptable, bioassay compatible level. These extracts were then spiked with various concentrations of digoxin, 3, 12-diacetate digoxigenin, digoxigenin, gitoxin and gitoxigenin, *i.e.* compounds which not only differ from each other with respect to their affinity constant of the affinity interaction, but also in ionisation efficiency, meaning MS-detectability. Using these spiked  
20 samples the detection limits of the selected compounds were determined in a large set of natural extracts.

With respect to digoxin the minimum detection limit was determined to be approximately 6 pmol in higher plant extracts. Although some variation in detection limits was measured, due to matrix effects of the  
25 different sample types, the maximum detection limit was determined to be 13 pmol.

In addition, all other compounds could be detected in the natural extracts as well. Due to lower ionisation efficiencies with respect to digoxin, digoxigenin and gitoxigenin proved to be hardest compounds to detect. This is  
30 reflected by the detection limits of these compounds which were found to be 25-30 pmol.

**Data evaluation** The procedure used to evaluate the collected data, as described in the experimental section, proved to be highly suitable in making unbiased assignments of bioactive compounds. Using the background  
35 subtraction option provided by Xcalibur, background ions could be efficiently removed, thus facilitating bioactive compound recognition.

In case of the bioactive compounds, used to test the system under real life conditions, the corresponding  $m/z$  ratios could easily be detected using this form of data evaluation. Additional peaks, which seemed interesting at a first glance, could easily be discarded as either spikes or incompletely removed background ions.

By comparing the MS-spectra of a natural extract obtained after simple sample cleanup and after orphan bioassay analysis, the shear power of the method to rapidly detect bioactive compounds for orphan receptors in complex matrixes, is clearly demonstrated. As the first spectrum shows a multitude of compounds still present after sample cleanup, the second spectrum shows a clean MS-spectrum displaying only the  $m/z$  values of digoxin as the principal ions.

Claims

1. On-line detection method comprising the on-line coupling of the effluent of a fractionation step to a mass spectrometer, which method comprises the addition of a controlled amount of an affinity molecule to said effluent, whereby the affinity molecules bind analytes in the effluent, followed  
5 by a separation step using a restricted-access support, whereby the analyte-affinity molecule complex is permeated, followed by a suitable dissociation step to dissociate the analyte-affinity molecule complex, followed by a second separation step in which the dissociated analyte and affinity molecules are separated, followed by detection of the analyte using the mass spectrometer.
- 10 2. On-line detection method according to claim 1, in which the second separation step is carried out using a restricted-access support, in which the affinity molecule is retained, followed by elution of the analyte from the restricted-access support using a suitable carrier stream, and directing the eluted stream to the mass spectrometer.
- 15 3. On-line detection method according to claim 1, in which the second separation step is carried out using a hollow fiber support, whereby the analyte is permeated and the permeate is directed to the mass spectrometer.
4. On-line detection method according to any of the preceding claims, in which the dissociation step is a low pH shock, contacting with a high ionic  
20 strength solution, contacting with an organic solvent and/or contacting with a chaotropic reagent.
5. Method according to any of the preceding claims in which the fractionation step is a liquid chromatography separation, a capillary electrophoresis step or a combinatorial chemistry system, which is optionally  
25 followed by a separation step which removes the high molecular weight background.

6. Method according to claim 5, in which the liquid chromatography separation step is a HPLC, a reversed phase HPLC, a CE, a CEC, a IEF or a MEKC step.

7. Method according to any one of the preceding claims, in which the  
5 mass spectrometer is of the type chosen from the group consisting of electrospray ionization type, atmospheric pressure ionization type, quadrupole type, magnetic sector type, time-off-flight type, MS/MS, MS<sup>n</sup>, FTMS type, ion trap type and combinations thereof.

8. Method according to any one of the preceding claims, in which the  
10 mass spectrometer is set to detect ions of a selected single m/z trace, selected multiple m/z traces, in scanning mode or any sequential mode.

9. The method of any of the preceding claims wherein the affinity molecule is an affinity protein.

10. The method of any of the preceding claims wherein the affinity  
15 molecule is an orphan receptor.

11. Compound detected by the method of any one of the preceding claims.

12. The use of a compound of the preceding claim as a ligand for affinity molecules.

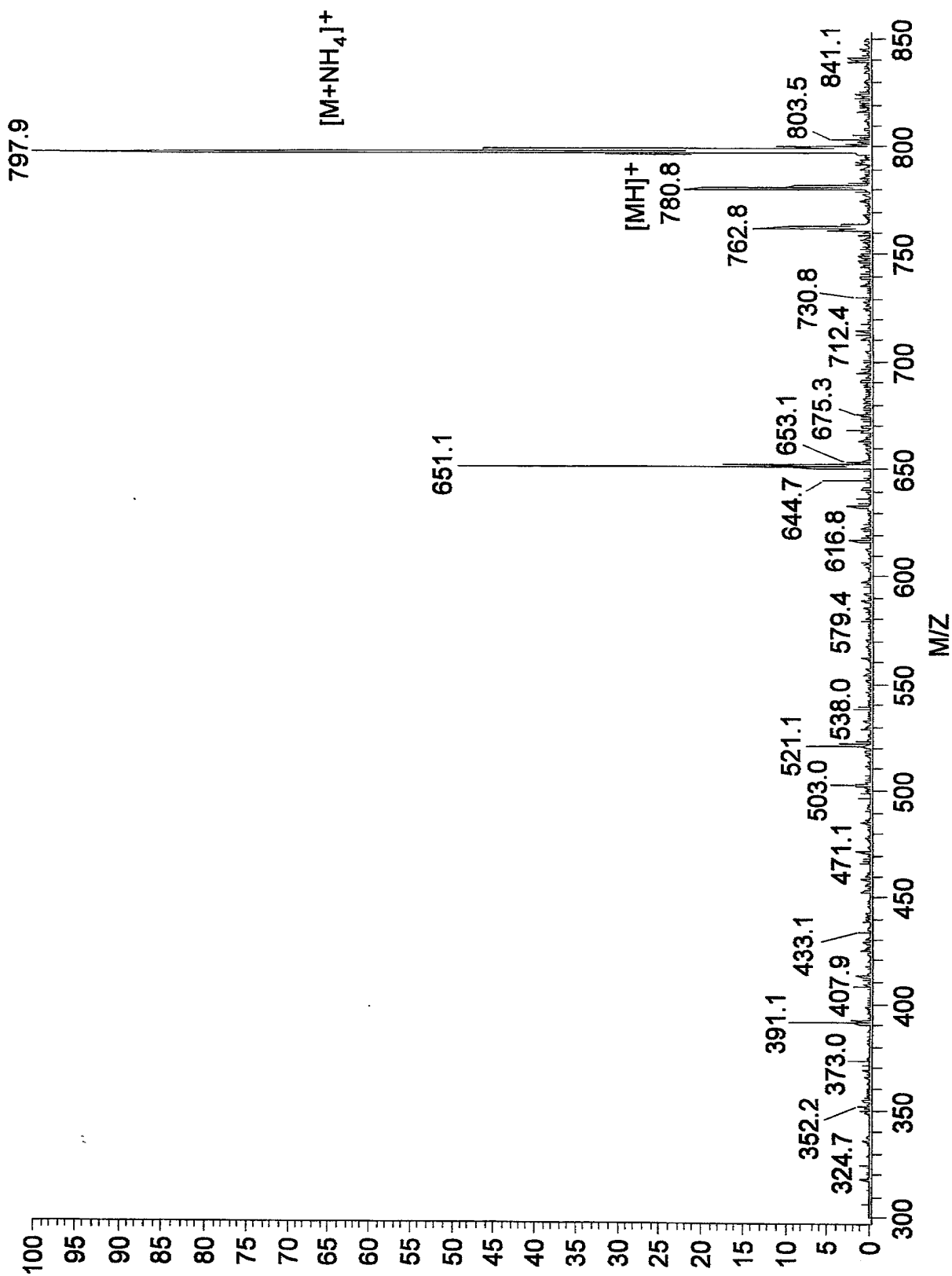


FIG. 1

09-980,646

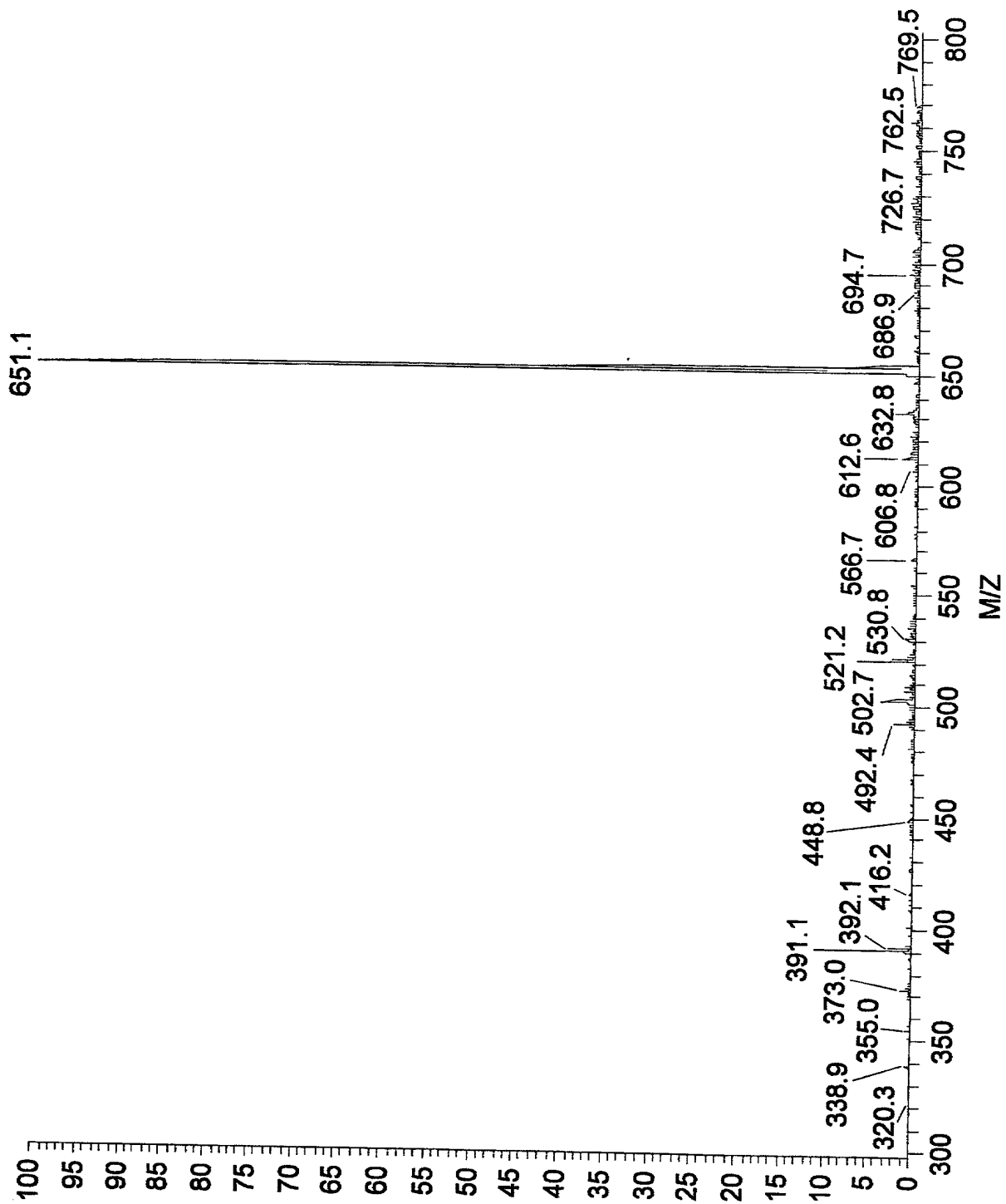


FIG. 2

## Power of Attorney

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Attorney	Registration Number
Bruce S. Londa	<u>33,531</u>
Lorimer P. Brooks	<u>15,155</u>
William R. Robinson	<u>27,224</u>
Kurt G. Brisco	<u>33,141</u>
William C. Gerstenzang	<u>27,552</u>
Robert A. Hyde	<u>46,354</u>
Davy E. Zoneraich	<u>37,267</u>
Mark A. Montana	<u>44,948</u>
Stephen G. Ryan	<u>39,015</u>

I hereby authorize them or others whom they may appoint to act and rely on instructions from and communicate directly with the person/organization who/which first sends this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instructed otherwise.

Please direct all correspondence in this case to at the address indicated below:

NORRIS, MCLAUGHLIN & MARCUS, P.A.  
20 Exchange Place, 37<sup>th</sup> Floor  
New York, New York 10005  
United States of America

Full Name of Sole or First Inventor		
Family Name van der Greef	First Given Name Jan	Second Given Name
Residence and Citizenship		
City of Residence Driebergen <i>NLY</i>	State or Country of Residence The Netherlands	Country of Citizenship The Netherlands
Post Office Address		
Street Address De Beaufortlaan 8	City Driebergen	State & Zip Code or Country 3971 BM
Signature of Inventor <i>[Signature]</i>		Date 2-11-2001

Full Name of Second Inventor, if any		
Family Name Irth	First Given Name Hubertus	Second Given Name
Residence and Citizenship		
City of Residence Amsterdam <i>NLY</i>	State or Country of Residence The Netherlands	Country of Citizenship The Netherlands
Post Office Address		
Street Address Rooseveltpaan 112-II	City Amsterdam	State & Zip Code or Country 1078 NP
Signature of Inventor <i>[Signature]</i>		Date

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

Prior Provisional Application(s)	
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date
Serial Number	Day/Month/Year Filing Date

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s), or under 35 U.S.C. §365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Prior U.S. or International Application(s)		
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)
Serial Number	Day/Month/Year Filed	Status (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



**Declaration and Power of Attorney Patent Application  
(Design or Utility)**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: 'Identification of ligands for orphan receptors using mass spectrometry'

the specification of which

- ☐ is attached hereto  
x was filed on October 26, 2001 as application serial no. 09/980,646  
and or PCT International Application number PCT/NL00/00268 and was amended  
on (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information know to me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 35 U.S.C. §365(b) of any foreign application(s) for patent or inventor's certificate, or 35 U.S.C. §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate of PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)		
Number 99201301.1	Country EP	Day/Month/Year Filed 26-04-1999
Number	Country	Day/Month/Year Filed
Number	Country	Day/Month/Year Filed